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LOCALIZATION OF INVERTASE IN A STRAIN OF YEAST

BY

J. FRIIS and P. OTTOLENGHI[1]

While cells grown in a medium containing glucose as the carbon source have only an intracellular invertase, sucrosegrown cells appear to have both an intracellular and an extracellular one. By digestion of the yeast cell wall with gastric juice of *Helix pomatia*, at least 74 per cent of the external invertase of adapted yeast can be removed. No change is observed after such treatment of unadapted cells.

During the process of adaptation to sucrose splitting there is a change in the localization of the cellular invertase. First of all sucrose is admitted into the cell (after a period of adaptation), and secondly, invertase is secreted through the cell membrane and remains trapped in the cell wall structure.

INTRODUCTION

The distribution of invertase activity in the yeast cell has been the subject of a great number of investigations. The evidence which has been obtained gives a strong indication that this enzyme is localized at, or actually outside, the cell membrane. The evidence for these conclusions arises from studies on the effects of pH variation on the *in vivo* and the *in vitro* invertase activity (16); on the effect of the uranyl ion as an inhibitor of invertase (4), and the reversal of this inhibition by phosphate ion concentrations considerably lower than those found intracellularly; on the distribution of hydrolysis products of sucrose in intracellular and extracellular space (4); on kinetic data which also take into consideration the permeability problems which are involved in such a study (1). From the last mentioned studies, by BEST, it was concluded that although the invertase must lie outside the cell membrane, a structural barrier was still present between the enzyme and the external medium, limiting the diffusion of substrate.

BURGER, BACON and BACON (2) have recently reported that treating baker's yeast with a "cellulase" preparation from the gut juice of *Helix pomatia*, for 8 hours at 20°C promoted the liberation of about 50% of the invertase activity of the cells without affecting their viability to an appreciable degree. All the invertase could be

[1] Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

released from the cells if they were exposed to ethyl acetate prior to the treatment with the cellulase preparation. Ethyl acetate treatment alone will liberate only very minor amounts of invertase (11). It was suggested by these observations (2), that even after ethyl acetate treatment a barrier remained preventing the exit of invertase from the cell structure. The barrier seemed to be removed upon treatment with "cellulase".

The finding that purified preparations of invertase still contained a large proportion of a carbohydrate material (a mannan with a minimal molecular weight of 7770) which appears to act as a stabilizer for the enzyme (6), as well as the existence of a mannan layer in the yeast cell wall, with which there is associated a protein component (13), makes the concept of the invertase being located in the cell wall of the yeast even more attractive.

It appears from the literature that usually when the splitting of sucrose into its component monosaccharides by a yeast, or yeast extract has been studied, the enzymatic activity being investigated has been termed "invertase". Most experiments which have been reported on the localization of such invertase activity in the yeast cell were carried out with baker's yeast (*S. cerevisiae*) from several different sources. Genetic analysis of one strain of *S. cerevisiae* (Yeast Foam) by WINGE and ROBERTS (19) has shown that there is a single gene, termed R_2, controlling the initiation of the fermentation of raffinose and sucrose, by what has been called a β-h-fructosidase. This same strain of *S. cerevisiae* also contains at least 3 genes which can independently control the fermentation of maltose (17). At least 2 of these genes are involved in the presence of systems fermenting sucrose as well as maltose even in cases where R_2 is absent (7, 18, 20). It is therefore necessary to exercise some caution in the interpretation of results obtained with yeasts which may contain a multiplicity of enzymes acting in a similar manner and on the same substrate.

In the present paper the term "invertase" shall describe any sucrose splitting activity, regardless of molecular specificity.

MATERIALS AND METHODS

The yeast used in these studies was a single spore culture, number 303–3 from the Carlsberg collection. This homozygous diploid strain which is derived from hybridizations involving *S. carlsbergensis*, *S. chevalieri*, and *S. italicus* (*Gilliland*) (20), has the single R_2 gene for invertase and is completely recessive for the known genes for maltose and melibiose fermentation.

The yeast was grown at 26°C for approximately 10 hours, in either 2 % glucose-yeast water or in 2 % sucrose-yeast water, as described in the experimental section. The cells were harvested by centrifugation and were then very thoroughly washed by repeated suspensions in large volumes of distilled water, and subsequent centrifugation.

Protoplasts were prepared according to the method described by EDDY and WILLIAMSON (5), slightly modificd. According to these authors the protoplasts are stable for several hours in 0.5 M rhamnose. We found that most of the cells will lyse in this medium. They will, however, remain stable for several days, if kept in solutions of 1.2 M rhamnose, or in 0.6 M NaCl. The NaCl, as well as maintaining the integrity of the protoplasts by virtue of the osmotic pressure created, also prevents the growth of both protoplasts and normal cells[1] even when a nitrogen source (yeast water) and a carbon source (e.g. glucose) are provided. The yeast cells were suspended in the "cellulase" preparation, which was made up by dissolving 10 mg of a lyophilyzed powder of gut juice from *Helix pomatia* (21), in 1 ml of 0.6 M NaCl containing 0.02 M phosphate at pH 5.5. Two to three hours of incubation at room temparature were sufficient to liberate at least 90 % of the cells from their cell walls. After the snail enzyme treatment the cells were again washed several times by suspension and centrifugation in the NaCl-phosphate medium, in order to remove all traces of contaminating snail enzyme preparation and cell debris. Control samples of normal yeast cells were incubated for the same period of time in a medium containing the NaCl-phosphate but no snail enzyme.

The fermentation of sucrose (CO_2 production) by our cell suspensions could not be employed as a method for the quantitative assay of the invertase activity since the rate limiting reaction system occurs subsequent to the splitting of sucrose.

The invertase assay actually used was based on linking the invertase activity to oxygen consumption by means of glucose oxidase. However, since yeast cells are capable of utilizing glucose, as far as known, via hexokinase and the Embden-Meyerhof pathway, fructose, which is also a substrate for hexokinase, was included in the assay system to saturate the hexokinase and thus to inhibit the utilization of glucose by this particular system. Under these

[1] Yeast cell which have not undergone treatment with the digestive enzyme preparation, will be referred to as normal cells, as opposed to the protoplasts.

conditions any glucose formed by the splitting of sucrose will be taken care of exclusively by the externally added glucose oxidase.

The assay system, which was essentially that described by KAPLAN (9), consisted of the cell preparation to be assayed, glucose oxidase (3 mg per ml), catalase (3 μl per ml), ethanol (7.7×10^{-2} M), fructose (0.1 M), sucrose (3.3×10^{-2} M), all suspended in 0.6 M NaCl containing 0.02 M phosphate at pH 5.5. All concentrations are final concentrations. In later experiments the catalase was omitted as it was not found to produce any effect on the rate or on the total oxygen consumption.

Measurements were carried out with the Cartesian divers as described by HOLTER and LINDERSTRØM-LANG (8) and WATERLOW and BURROW (14). The divers had a gas volume of between 6 and 10 μl, and the reaction mixture was in a total volume of 0.5 μl. The CO_2 produced by the cellular respiration was absorbed by 0.6 M NaOH. This concentration, being the same as the NaCl concentration in the reaction mixture, was chosen so as to avoid interference by distillation from the NaOH compartment to the reaction mixture, or vice versa. The experiments were carried out at a temperature of 20.4°C. In anaerobic experiments the atmosphere was of 5% CO_2 + 95% N_2.

In the invertase assays, the procedure employed involved pipetting 10 μl each of a: the cell suspension, b) a fructose, or fructose + glucose solution, and c) the glucose oxidase, ethanol, and catalase (when used). After mixing, a 0.5 μl portion was transferred to the diver. The method has been found to give reproducible results, as can be judged from Figure 2a (upper curve), for which two divers were filled with identical reaction mixtures prepared, however, in two distinct series of pipettings of the components.

In the assays, apart from a reagents blank (all reagents with the exception of the cell preparation), the rate of oxygen consumption by the cells in the presence of fructose but without sucrose was also measured. As some loss of cellular material invariably occurs during the "cellulase" treatment, due to lysis of some of the cells, it was necessary to find a way of comparing the cellular concentration in samples of protoplasts with the control samples of normal yeast cells. The method which was used for this purpose, consisted of reducing the rates of oxygen consumption induced by the addition of fructose to protoplasts and to normal cells, to a common value.

This was done by multiplying the rate of oxygen consumption by normal cells when in the presence of fructose by the factor:

$$\frac{O_2 \text{ uptake with fructose, by protoplasts}}{O_2 \text{ uptake with fructose, by normal cells}}$$

and multiplying the rate of oxygen consumption of the normal cell preparation containing both fructose and sucrose by this same fraction.

Example:

a. O_2 consumption: normal cells + fructose = 10.8×10^{-3} μl/hr.

b. O_2 consumption: protoplasts + fructose = 15.0×10^{-3} μl/hr.
ratio b/a = 1.39.
corrected O_2 consumption for normal cells + fructose
$10.8 \times 10^{-3} \times 1.39 = 15.0 \times 10^{-3}$ μl/hr.

c. O_2 consumption: normal cells + fructose + sucrose = 31.2×10^{-3} μl/hr.

d. O_2 consumption: protoplasts + fructose + sucrose = 21.6×10^{-3} μl/hr.
corrected O_2 consumption for normal cells + fructose + sucrose =
$31.2 \times 10^{-3} \times 1.39 = 43.3 \times 10^{-3}$ μl/hr.
Invertase activity for normal cells = $(43.3 - 15.0) \times 10^{-3}$ μl/hr.
$= 28.3 \times 10^{-3}$ μl/hr.
Invertase activity for protoplasts = $(21.6 - 15.0) \times 10^{-3}$ μl/hr. = 6.6×10^{-3} μl/hr.

It was here assumed that the mechanisms involved in the utilization of oxygen in the metabolism of fructose are not affected by the removal of the cell wall. This assumption seems to be borne out by the results which will be presented in the next section.

The pipettes used were the Carlsberg constriction pipettes.

Inorganic chemicals used were all analytical reagents from B.D.H., Merck-Darmstadt, or Riedel-de Haën. Sugars were from either Pfanstiehl or B.D.H., and were used without further purification. The glucose oxidase used was a crude preparation donated by Professor DETLEV MÜLLER of the Plant Physiology Department at the University of Copenhagen, and the catalase was a purified preparation, a gift of Professor HUGO THEORELL of the Karolinska Institute, Stockholm.

RESULTS

It became apparent at an early stage of this investigation that the conditions under which the yeast was maintained during the growth period prior to its use, were of considerable importance in

determining the results. If the cells were grown in a yeastwater medium containing glucose as the sole carbon source, no invertase could be measured (Figs. 1a and 1b) by means of the assay system employing glucose oxidase, as described under "Methods". If, on the other hand, the yeast was grown in the presence of sucrose as

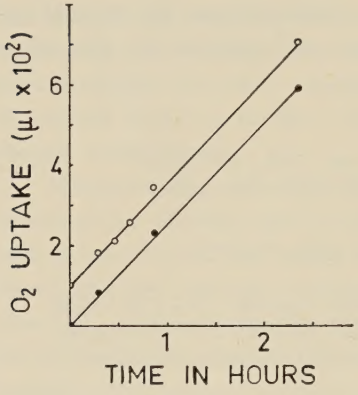

Fig. 1a.

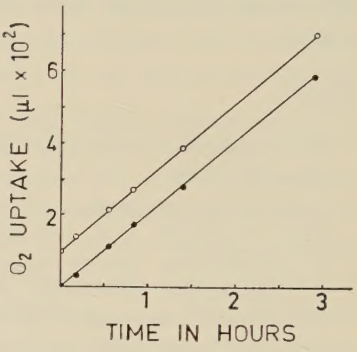

Fig. 1b.

Fig. 1a. Non-adapted yeast: The measurement of invertase activity in normal cells. •—• with fructose; o—o with fructose and sucrose. Other experimental conditions were as described under "Methods". One of the curves has been displaced for clarity.

Fig. 1b. Non-adapted yeast: The measurement of invertase activity in protoplasts. •—• with fructose; o—o with fructose and sucrose.

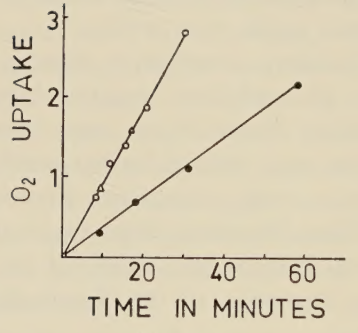

Fig. 2a.

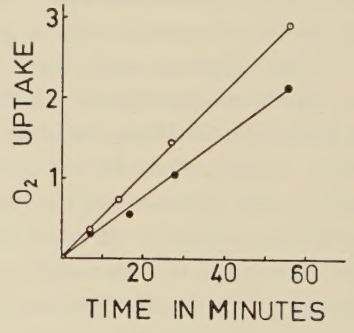

Fig. 2b.

Fig. 2a. Adapted yeast: The measurement of envertase activity in normal cells. •—• with fructose; o—o and △—△ with fructose and sucrose (duplicate experiment). O_2 is in μl/hr ($\times 10^2$).

Fig. 2b. Adapted yeast: The Measurement of invertase activity in protoplasts. •—• with fructose; o—o with fructose and sucrose. O_2 uptake is in μl/hr ($\times 10^2$).

the carbon source, invertase activity was measurable under our conditions (Figs. 2a and 2b).

Figs. 1a and 1b show the sucrose splitting activities of non-adapted cells measured as oxygen uptake. It is quite clear that the addition of sucrose to the normal cells (1a) or the protoplasts prepared from such cells (1b), produces absolutely no increase in the oxygen consumption when compared to the activity of the respective controls containing no sucrose. This demonstrates the absence of invertase activity in intact glucose-grown cells, but not the absence of invertase enzyme (see below).

In sucrose-grown cells, invertase activity can be demonstrated as seen in Fig. 2a. Although som invertase activity is still present

Table 1.

The invertase activity of intact, adapted normal cels and protoplasts (in μl O_2 uptake per hour).

	Normal cells	Protoplasts	△
i	32.8×10^{-3}	8.4×10^{-3}	74 %
ii	6.1×10^{-3}	0.5×10^{-3}	92 %
iii	28.3×10^{-3}	6.6×10^{-3}	78 %

△ = invertase activity removed from cell by the digestion of the cell wall.

in protoplasts from these cells, at least 74% of it has been lost concomitantly with the removal of the cell wall (Fig. 2b and Table I). It is evident from these results that a large proportion of the invertase activity of the yeast cell can be removed together with the cell wall, by treatment with the preparation of snail gut juice.

When protoplasts are broken by freezing (in an ethanol-solid CO_2 mixture) and thawing, invertase in large concentrations can be found in the suspension whether cells were adapted to sucrose or not. The following problem thus arose: Assuming that the invertase in the unadapted cells is present in an active form, is the absence of activity due to the substrate not getting to the enzyme, or to the inability of the enzyme to reach the substrate? An alternative would be the presence, in the cellular regions attacked by the snail's digestive enzymes, of an inactive enzyme, capable of being activated by the substrate, or somehow, by freezing and thawing.

In order to study these questions the total invertase in the cells was measured before and after removal of the cell wall, in both adapted and non-adapted cells. The normal cells (as well as the protoplasts) were frozen and thawed five times in succession, a process

which makes the cells quite permeable to external substrates (10). The results in Table II show that in the adapted cells the amount of invertase lost from the cells (as measured after disruption of the cells by freezing and thawing), concurrent with the removal of the cell

Table 2.

The invertase activity in intact and disrupted normal cells and protoplasts (in $\mu l \times 10^{-3}$ O_2 uptake per hour).

	Untreated			Frozen and thawed		
	Normal	Protoplasts	△	Normal	Protoplasts	△
Adapted cells	28.3	6.6	21.7	38.7	16.7	21.8
Non adapted cells.....	—	—	—	21.9	23.2	1.3 (+ 5 %)
				26.0	22.4	3.6 (− 14 %)

△ = change in invertase activity brought about by the digestion of the cell wall.

wall, corresponds to the decrease in enzyme activity shown by the intact cells on becoming protoplasts. In other words there has seen an actual removal of cellular invertase with the cell wall, obviating the possibility that the effect observed is due to an interference by the *H. pomatia* digestive juice with a mechanism for the transport of substrate across a barrier to intracellular enzymatic sites. The presence of an inactive invertase, capable of being activated by either substrate or by freezing and thawing is also excluded. It can also be seen from Table II that the removal of the cell wall from non-adapted cells, produces, if one takes into account the number and nature of the various manipulations involved in the assay, no very significant change in the total invertase content of the cells; that is to say, the amount of invertase in the normal cells is the same as that in the protoplasts. This is evidence for the absence of invertase from regions of the cell which are affected during the digestion process.

As mentioned under "Methods", to permit the comparison of enzyme activities of normal cells and of protoplasts, the results given in Tables I and II have been adjusted for the cellular concentration of the samples of normal cells and of protoplasts made from them. This correction was made on the basis of the oxygen consumption induced by the addition of fructose to the cell suspensions,

and assuming that the process involved in this oxygen consumption was unaffected by the removal of the cell wall. The agreement obtained when comparing the difference in the amounts of invertase in adapted, normal cells and protoplasts, when intact and after disruption, and the agreement in the total amounts of invertase found in whole cells and protoplasts of non-adapted cells (Table II) indicate the assumption made is valid.

We feel that in the results obtained when comparing the amount of invertase in disrupted non-adapted normal cells and protoplasts (Table II), the divergences of + 5 % and — 14 % are not really significant when they are considered together with the rest of the experimental evidence presented.

When adapted cells suspended in NaCl-phosphate medium, are brought into contact with sucrose under anaerobic conditions, a constant rate of fermentation of the substrate as measured by an output of CO_2, begins without delay. This is not true, however, with non-adapted cells. In the latter case a definite lag period (of varying duration) has been repeatedly found to be required before activity reaches a maximum (Fig. 3)[1].

In the experiment shown in figure 3 aliquots were taken before the addition of sucrose and after 7 hours of incubation with sucrose. After washing the cells, invertase assays were performed on these two samples in the usual manner in the presence of 0.1 M fructose. No measurable invertase activity was found in either case. Furthermore, in an experiment essentially the same as that shown in Fig. 3, the total invertase in the cells was measured (in frozen and thawed preparations), before and after one hour of incubation with cusrose. No change in the amount of invertase was observed to have taken place, even though the rate of CO_2 production had increased about four fold.

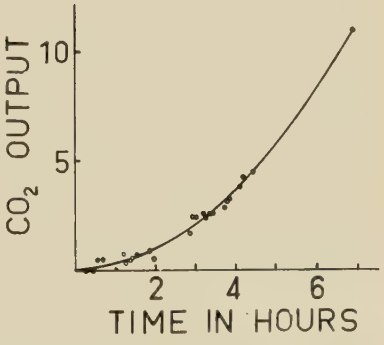

Fig. 3. The fermentation of sucrose by non-adapted yeast. CO_2 output is in μl ($\times 10^2$). The cells were suspended in the NaCl-phosphate solution supplemented with 0. 01 M sucrose. The reaction was started at zero time by the addition of substrate form a side-drop.

It appears, therefore, that

[1] This experiment has been carried out also in the absence of NaCl. The results did not differ from those obtained in its presence.

when resting yeast is incubated with sucrose, it is capable, after a period of adaption[1], of taking up the sucrose and fermenting it. This agrees with the report of MYRBÄCK and WILLSTAEDT (12) in which it was shown that after destruction of the external invertase by treatment of the yeast with acid, the cells were still capable of fermenting sucrose. The absence of invertase activity as measured by means of our assay can be construed as an indication that fructose in relatively high concentrations (0.1 M) will inhibit the penetration of sucrose into the yeast cell, probably by interfering with some transport mechanism. DAVIES (3), using *S. fragilis*, also has shown that

Table 3.

Invertase activity in intact, normal yeast (non-adapted) incubated with sucrose-yeast water[1] as a nitrogen source.

Incubation time	Net invertase
0 mins.	$(0.2 \times 10^{-3}$ µl/hr)[2]
7 mins.	1.6×10^{-3} µl/hr
15 mins.	2.0×10^{-3} µl/hr
60 mins.	4.8×10^{-3} µl/hr

[1] All values were corrected for cellular concentration from a calibrated standard curve of yeast concentration against an optical density at 500 mµ as read in a BECKMAN D. U., using cuvettes of 1 cm light-path.

[2] Not significantly different from zero.

a short adaption period was required before glucose-grown cells would begin to ferment sucrose. Both glucose and fructose, when in high concentrations, were shown to inhibit this adaption.

The results which are obtained when cells are suspended in a medium containing both a nitrogen source and sucrose are rather different from those just mentioned above. Invertase was measured in cells incubated at 20°C in yeast water containing 0.02 M sucrose, for 7, 15, and 60 minutes, as well as in a pre-incubation sample. As soon as the aliquots were obtained, they were cooled in an ice-bath, and the cells were washed repeatedly with cold NaCl-phosphate solution. The assays were then carried out as usual. The results in Table III indicate that the invertase has become accessible to sucrose, even when measured in the presence of fructose, presumably indicating a change in localization of part of the enzyme.

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[1] In using this term, no specific mechanism is implied for the changes which have apparently taken place.

DISCUSSION

From the experiments involving the removal of the cell wall it has become evident that if cells are grown with glucose as the carbon source, no invertase is present in those portions of the cell which are removed by the gastric juice of *Helix pomatia*. On the other hand, in cells which have grown in the presence of sucrose some invertase is to be found bound to the cell wall material.

As is to be expected from what is known of the sites of protein synthesis, invertase is essentially an intracellular enzyme, whether the yeast has been adapted or not. During adaptation a stepwise process, involving a preliminary uptake of sucrose into the cell, followed by a subsequent secretion of invertase, takes place. WICKERHAM (15) has shown that in many yeast strains (but not in all) a secretion of invertase occurs into the surrounding medium. We have observed the same phenomenon with the yeast strain used in this study, both with normal cells and with protoplasts prepared from them, but unlike WICKERHAM, we cannot rule out the possibility that at least part of the "secreted" enzyme has come from dead cells.

It is assumed that the cell membrane acts as a barrier through which sucrose is not free to pass. The initial period of adaptation required before fermentation of sucrose attains a maximum rate in non-growing, non-adapted cells, and the inhibition of such a process by fructose, are indications that the entry of sucrose into the cell does not occur by free diffusion. The increasing slope of Fig. 3, implies an increase in the permeability coefficient for either sucrose or invertase. However, since in the experiment represented by Fig. 3 the outward movement of invertase has, as already mentioned, been excluded, it is concluded that the changes in permeability here refer to the transport of sucrose. Also the cell wall seems to act as a barrier, in this case to the free movement of invertase molecules. When invertase is secreted from the cellular interior it remains trapped (at least partly) in the structural framework of the cell wall, thus creating a physiologically advantageous concentration of the enzyme. The efficiency of the cell wall as a barrier of the above type depends presumably also on the strain of yeast, giving rise to the variations in the capacity of different yeasts to secrete invertase (15).

It is suggested, therefor, that the adaptation to sucrose cleavage by invertase involves, eventually relocation of this enzyme from intracellular to extracellular sites. The stimulus for this process

can be supplied by the substrate, sucrose, after a preliminary adaptation of the cell to admit sucrose. Although the cell is capable of splitting sucrose both intracellularly and extracellularly, it is possible that in adapted yeast the invertase in the cell wall region is present in concentrations sufficient to hydrolyse any added sucrose before the sugar has a chance to reach the interior of the cell.

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LOCALIZATION OF MELIBIASE IN A STRAIN OF YEAST

BY

J. FRIIS and P. OTTOLENGHI[1]

Melibiase has been found in both adapted and non-adapted cells, provided the permeability properties of the cells are first altered by freezing and thawing the cells.

During adaptation, the cells at one stage become capable of fermenting melibiose without any apparent melibiase activity in the external regions of the cell. Adapted cells, on the other hand, have melibiase activity apparently located in the cell wall. This activity can be completely removed upon digestion of the cell wall with a preparation of digestive juices from *Helix pomatia*. Adapted cells and protoplasts prepared from them are capable of secreting melibiase into the surrounding medium, while protoplasts prepared from non-adapted cells cannot do so.

INTRODUCTION

As a companion to the study of invertase (2) we have undertaken a similar investigation on melibiase.

Some of the enzymological characteristics of a yeast melibase have been reported by ADAMS, RICHTMYER and HUDSON (1) who found it as a contaminant in their purified preparations of invertase. In *Saccharomyces carlsbergensis*, SPIEGELMAN, LINDEGREN and LINDEGREN (12) found that the melibiase activity is controlled by a single dominant gene, a finding which is confirmed by GILLILAND (4). According to LINDEGREN, SPIEGELMAN and LINDEGREN (9), the melibiase of *S. carlsbergensis* is an adaptive enzyme, which de la FUENTE and SOLS (3) suggest is located in contact with the external environment of the cell, in such a way that the metabolism of melibiose by the yeast is initiated by the splitting of melibose outside the cell, followed by the uptake of the monosaccharides, and their intracellular fermentation. There is no real evidence for the fermentation of melibiose by a mechanism not involving the initial cleavage of the disaccharide into its constitutive hexose sugars (5, 6).

[1] Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

MATERIALS AND METHODS

The strain of *Saccharomyces carlsbergensis* used in this investigation was strain number 982 from the Carlsberg collection (it was obtained in 1946 from Dr. C. C. Lindegren, Southern Illinois University, Carbondale, USA).

The yeast was normally grown overnight in glucose-yeast water and was then harvested if it was to be used as a source of non-adapted cells. If adapted cells were required, the yeast was grown for a further period of 6 hours in yeast water containing melibiose, raffinose or galactose, as it was found that all three sugars adapted the yeast to splitting melibiose. Melibiose itself was routinely used, however (7).

The yeast, after harvesting, was washed with distilled water and was then suspended in 0.6 M NaCl containing 0.02 M phosphate at pH 5.5. The assays of melibiase were performed by either an anærobic method or by an ærobic one. Anærobically, the assays were carried out (unless specified otherwise in the experimental section) in the Cartesian diver respirometers in an atmosphere of 5% $CO_2 + 95\%$ N_2, at 20.4 °C, estimating the CO_2 evolution due to the fermentation of added melibiose.

The ærobic method was better adapted to quantitative measurements and was based on the rate of oxygen uptake produced by the action of glucose oxidase on the glucose liberated by the splitting of melibiose by the yeast cells. The assay system was analogous to that described in (2), and consisted of the cell preparation to be assayed, fructose (0.1 M), melibiose (3.3×10^{-2} M), glucose oxidase (3 mg per ml), ethanol (7.7×10^{-2} M), all in 0.6 M NaCl containing 0.02 M phosphate at pH 5.5. The rate of oxygen consumption in the absence of cells was the blank subtracted from the experimental values. The activities measured by either one of the above methods will be considered as due to melibiase. Measurements were carried out with Cartesian divers.

When comparing the melibiase activities in normal yeast[1] and protoplasts, as the cellular concentrations in the two samples differed, they were adjusted as described in (2). This correction was based on the oxygen uptake in the presence of fructose, but not of melibiose, in the glucose oxidase system, assuming that no quantitative alteration in the mechanism for the oxidation of fructose takes place in the yeast cell during the removal of the cell wall.

[1] Normal cells, as opposed to protoplasts, are yeast cells which have not undergone treatment with the *Helix pomatia* gut juice preparation (13).

The preparation of the protoplasts by the digestion of the cell wall with a preparation from the digestive juices of *Helix pomatia*, is reported elsewhere (2, 7).

At the end of experiments which had lasted many hours, the cell suspension was examined microscopically. In none of the experiments to be reported was either contamination, or breakage of protoplasts, detectable.

Inorganic chemicals used were all analytical reagents from B.D.H., Merck-Darmstadt, or Riedel-de Haën. Sugars were from either Pfanstiehl or B.D.H. and were used without further purification. The glucose oxidase used was a crude preparation donated by Professor DETLEV MÜLLER of the Plant Physiology Department at the University of Copenhagen.

RESULTS

As was true for invertase (2) and as had been found earlier by LINDEGREN *et al.*(9), the medium in which the yeast was grown was found to determine the presence or absence of melibiase activity in intact *S. carlsbergensis*.

A NaCl-buffer suspension of washed, adapted yeast would show an immediate output of CO_2 upon coming into contact with melibiose under anærobic conditions, the rate of gas evolution remaining constant with time. With non-adapted cells suspended in NaCl-buffer medium, melibiase activity, as measured by an evolution of CO_2, could be only found after a period of adaptation, the duration of which varied from experiment to experiment. A typical curve is shown in Fig. 1.

Although we have not made any quantitative comparisons on a per cell basis, melibiase is measurable, without a delay in the attainment of a maximum rate, in both adapted and non-adapted cells, provided the cells are first rendered permeable to the substrate by freezing and thawing them (8).

When non-adapted normal cells are suspended in NaCl-phosphate supplemented with melibiose, they become capable of fermenting this disaccharide after a short period of adaptation, as was shown in Fig. 1. Measurements of melibiase by the other method *i. e.* the glucose oxidase system, failed to reveal any melibiase activity in samples taken both at the time of addition of the sugar (when the fermentation rate was zero), and after 3 hours of incubation

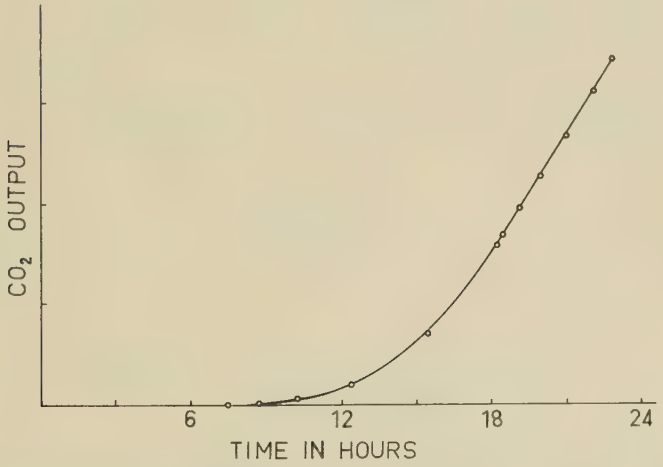

Fig. 1. The anærobic adaptation to the fermentation of melibiose, measured in Warburg respirometers with 3×10^{-2} M melibiose, 10^{-2} M phosphate at pH 5.5, and 0.6 M NaCl, at a temperature of 30° C and in an atmosphere of 5% CO_2 + 95% N_2.

with melibiose (at which time the fermentation of melibiose was proceeding at a constant rate).

As discussed in the previous paper (2), it seems likely that also in this case the fructose used in the glucose oxidase assay, inhibits the uptake of melibiose by the cells so that this assay procedure would essentially measure external melibiase activity. Thus the negative results obtained with this system need not necessarily contradict those obtained with the fermentation assays. It is probable that one of the effects of adaptation as just described, is to permit the substrate to reach the enzymatic sites within the yeast cell thus allowing fermentation to proceed.

Since in the case of invertase a further effect was the transfer of the enzyme from an intracellular to an extracellular location, this possibility was also investigated for melibiase, by means of protoplasts.

When adapted cells have their cell wall removed by enzymic digestion with the snail gut juice preparation thus forming protoplasts, they are no longer capable of fermenting melibiose. Fig. 2 shows that although the fermentation of glucose proceeds quite readily no CO_2 evolution occurs at first; after a certain period of inactivity, however, the cells become once more capable of fermenting the

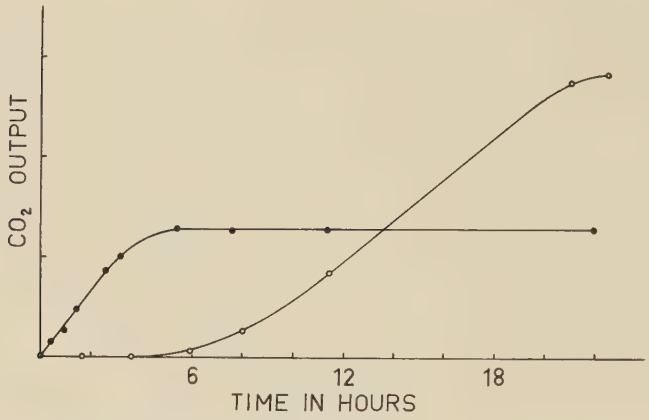

Fig. 2. The fermentation of glucose •—• and of melibiose o—o with protoplasts from adapted yeast, suspended in the NaCl-phosphate medium, supplemented with 6.6×10^{-3} M of either glucose or melibiose, measured in Cartesian divers. The leveling off of the curves is due to exhaustion of substrate.

disaccharide. The period of inactivity varied widely in duration, from one experiment to the other (3 to 13 hours), but the readaptation process remained essentially unchanged in character even when the protoplast suspension was supplemented with yeast water, or with yeast water and glucose, (Fig. 3). If the protoplasts were prepared from non-adapted cells, no adaptation to the fermentation of melibiose occured during incubations with the disaccharide of up

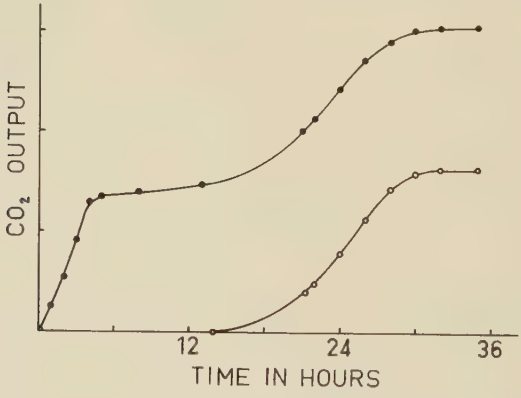

Fig. 3. Melibiase activity of protoplasts from adapted yeast, with melibiose (6.6×10^{-3} M) and yeast water o—o and with melibiose (6.6×10^{-3} M), yeast water and glucose (6.6×10^{-3} M) •—•. In the latter the initial evolution of CO_2 corresponds to the fermentation of the glucose. (Cartesian divers were used).

to 24 hours duration. It should be mentioned here that even in these long term experiments the protoplasts did not show any morphological changes.

Table I compares the measurements of melibiase activity made on yeast cells and protoplasts, both intact and after disruption by

Table 1.

The melibiase activity measured with the glucose oxidase system, in disrupted and intact normal cells and protoplasts (adapted). In μl O_2 uptake per hour.

Untreated			Frozen and thawed		
Normal	Protoplasts	Δ	Normal	Protoplasts	Δ
27.1×10^{-3}	—	27.1×10^{-3}	37.9×10^{-3}	12.9×10^{-3}	25.0×10^{-3}

Δ = decrease in the melibiase activity brought about by the removal of the cell wall.

The values in the table have been adjusted for the difference in the cellular concentrations in the samples of normal cells and protoplasts, as described under „Materials and methods".

freezing and thawing. Concidering the intact cells first, the loss of melibiase activity coincident with the removal of the cell wall was within 8% of the value for the decrease in enzyme from the total melibiase pool as assayed in the disrupted preparations. This difference of 8% which is not considered significant, does not prevent the conclusion that the enzyme lost must have been exposed to the digesting action of the digestive enzymes of the snail.

If protoplasts from originally adapted cells are incubated with 6.6×10^{-3} M melibiose, in the NaCl-phosphate medium, under anærobic conditions, at 20°C, for 36 hours, melibiase activity can be recovered in the supernatant fluid after removal of the cells by centrifugation. This same effect is observed with normal cells, but not when protoplasts from non-adapted cells are used. With the latter cell preparation, protoplasts were incubated under the same conditions employed for the protoplasts from adapted yeast, for a period of 42 hours, without any measurable melibiase activity being recovered in the supernatant. These assays were carried out with the glucose oxidase system. The results indicate that no appreciable breakage of cells had taken place, since this would produce the liberation of intracellular melibiase into the surrounding medium. Microscopic examination of the preparation showed the presence neither of cellular debris nor of any bacterial contaminants.

The readaptation to melibiose fermentation of the protoplasts from adapted cells probably involves the secretion of some material which is either soluble or else very firmly bound to the surface of the protoplasts, since washing the cells after readaptation has taken

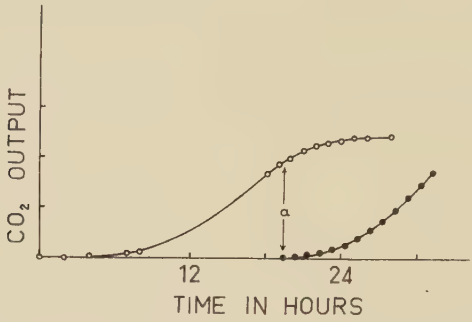

Fig. 4. Adaptation to fermentation of melibiose (6.6×10^{-3} M) by protoplasts from adapted cells o—o. At time (a) a sample incubated under identical conditions was washed with NaCl-phosphate medium, and was then resuspended at the same concentration with 6.6×10^{-3} M melibiose for the assay of melibiase •—•. (Cartesian divers were used).

place, temporarily abolishes the newly established fermentation processes (Fig. 4); the recovery of the capacity to ferment melibiose is now more rapid than it was the first time.

The secretion of melibiase is not the whole answer to the effect shown in Fig. 4. Some preliminary experiments seemed to indicate that when the supernatant from an incubation of protoplasts from adapted cells, with melibiose, was added to another batch of freshly prepared protoplasts, also from adapted yeast, an output of CO_2 began immediately. The rate of CO_2 output was greater than was to be expected from the observed melibiase activity of the supernatant. Furthermore, the melibiase-free supernatant from an incubation of melibiose with protoplasts from non-adapted cells also decreased very considerably the time required for the readaptation of protoplasts freshly prepared from adapted cells, when compared to the time required for readaptation in the absence of added supernatants.

DISCUSSION

The results obtained in this investigation are indicative that a similar system operates in the case of melibiase as was earlier reported for invertase (2).

Saccharomyces carlsbergensis, whether adapted to melibiose or not, has been found to posses melibiase activity, provided the cellular integrity is disrupted. However, unless the cells are grown in a medium

producing adaptation, the intact cells will not, at first, show any melibiase activity.

It is suggested that the process of adaptation can be subdivided into two phases: in the first stage melibiose is admitted into the cell where it can be fermented, without any evidence of melibiase activity existing on the exterior of the cell, while in the second stage melibiase is actually secreted from the cell interior. Although some melibiase finds its way into the surrounding medium, a part of it remains trapped in the cell wall and can be liberated by the removal of the cell wall itself. This picture is essentially the same as that described for invertase. A difference exists, however, between the melibiase and the invertase studied. In the case of invertase, as much as a quarter of the activity found in normal, intact cells, was still present in the naked protoplasts. With melibiase, complete removal of the enzyme follows the digestion of the cell wall material. This difference could be interpreted as a difference in the localization of the two enzymes, *i. e.* invertase could be more strongly bound to some inner layer of the cell wall, a layer which may remain behind when the bulk of the cell wall undergoes digestion (10, 11, 7) or the difference may lie in a greater susceptibility of the melibiase to attack and inactivation by some enzyme present in the digestive juice of the snail.

The behaviour of protoplasts in the presence of melibiose, suggests the possibility that a mechanism for the secretion of melibiase is triggered off sometime during the original adaptation of the normal yeast cells. This mechanism remains active even after the removal of the cell wall of the yeast, with the formation of the protoplasts. If protoplasts are prepared from non-adapted cells in which the hypothetical mechanism promoting melibiase secretion was inactive, the protoplasts remain unable to secrete the enzyme, and for that matter, remain also unable to ferment melibiose.

It would be of interest to investigate, in a similar manner to that employed here, the behaviour of maltase of yeast, which according to de la FUENTE and SOLS (3), is an intracellular enzyme, requiring an adaptable system for the transport of the substrate across the cell membrane to the enzymatic sites.

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